On page 14, line 30 through page 15, line 7 please substitute the paragraph with,

AK

"Thirteen founder mouse lines are generated and crossed with wild-type, B6/DBA2 mice. The resulting mice from each line are screened by PCR using tail DNA. Briefly, genomic DNA is extracted using a standard protocol, known to those in the art. PCR is run at 30 cycles: 93 °C x 30 seconds, 57 °C x 1.5 minutes, 72 °C x 5 minutes; using a sense primer (SEQ ID NO: 7) and an antisense primer (SEQ ID NO: 8)."

REMARKS

The Applicants have made amendments to the specification to change all instances of "Seq. Id. No." to "SEQ ID NO:" as specified in 37 C.R.F. 1.821(d). The Applicants submit that the amendments made are for the purpose of meeting format requirements and do not add new matter to the specification.

The Applicants submit that the sequence listings and specification are now in appropriate form to allow for examination of the application. If any issues remain regarding compliance with the sequence listing that may be expedited by a telephone conversation, please feel free to call the agent listed below, collect.

Respectfully submitted,

Dated: 22 October 2001

By: _____

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Docket No.: 6627-PA9013

[AMDYMASL01.I24]

INDICATION OF CHANGES MADE IN THE SPECIFICATION

Page 11, lines 16-29.

Initially, primer pairs, either a sense primer BSYNNDE, encoding amino acids 1-7 of β-synuclein ([Seq. Id. No.] SEQ ID NO: 1) and an NdeI site at its 5' end, and an antisense primer, corresponding to amino acids 73-83 of α-synuclein and amino acids 66-72 of β-synuclein ([Seq. Id. No.] SEQ ID NO: 2), or a sense primer, corresponding to amino acids 73-83 of α-synuclein and amino acids 73-79 of β-synuclein ([Seq. Id. No.] SEQ ID NO: 3) and an antisense primer BSYNNOT encoding amino acids 129-134 of β-synuclein and NotI site at its 5'end ([Seq. Id. No.] SEQ ID NO: 4) are incubated in PCR with plasmid pCEP4-human β-synuclein as a template. Individual PCR products are gel-purified and an aliquot of each product is combed with primers BSYNNDE ([Seq. Id. No.] SEQ ID NO: 1) and BSYNNOT ([Seq. Id. No.] SEQ ID NO: 4) to synthesize a full length of β-synuclein δ1 cDNA. The resulting PCR product is digested with NdeI and NotI and ligated to PROEX-1 previously digested with NdeI and NotI, finally to produce PROEX-1-β-synuclein δ1.

Page 11, line 30 through page 12, line 7.

PROEX-1-β-synuclein δ2 is similarly constructed by a two-step PCR protocol using PROEX-1-β-synuclein δ1 as a template. Initial primer pairs are either BSYNNDE ([Seq. Id. No.] SEQ ID NO: 1) and an antisense primer corresponding to amino acids 55-62 of β-synuclein and amino acids 63-72 of α-synuclein ([Seq. Id. No.] SEQ ID NO: 5) or a sense primer encoding amino acids 63-79 of α-synuclein ([Seq. Id. No.] SEQ ID NO: 6), and BSYNNOT ([Seq. Id. No.] SEQ ID NO: 4). For both of the newly-constructed plasmids, fidelity of the sequencing is confirmed using a commercially available delta taq cycle sequencing kit (Amersham).

Page 13, lines 12-22.

NACP/α-synuclein-specific polyclonal antibody (anti-NACP101-124) is raised against the synthetic peptide 'GKGEEGYPDEGILEDMPVDPGSEA' ([Seq. Id. No.] SEQ ID NO: 9) which is derived from residues 101-124 of NACP/α-synuclein. Immunoblotting analysis is performed as described above, the method of which is well known to those in the art. Briefly, each sample is resolved by SDS-PAGE (15%) and blotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a standard transfer apparatus. The membrane is then blocked with TBS containing 3% BSA, followed by an incubation with anti-NACP101-124 (1:1000) in TBS containing 1% BSA. The treated membrane is then incubated with ^{125}I-labeled protein A (ICN, Costa Mesa, CA), followed by autoradiography.

Page 14, lines 5-11.

Human α-synuclein is a 140 amino-acid molecule ([Seq. Id. No.] SEQ ID NO:10) that is encoded by a gene on chromosome 4 and was originally isolated from plaques of Alzheimer disease brains. Involved in synaptic function and neural plasticity, this 19 kD protein is a precursor of the highly hydrophobic 35 amino-acid metabolite, non-amyloid component (NAC). The NAC peptide can self-aggregate into fibrils and induces aggregation of the Aβ peptide.

Page 14, line 28 through page 5 line 2.

Thirteen founder mouse lines are generated and crossed with wild-type, B6/DBA2 mice. The resulting mice from each line are screened by PCR using tail DNA. Briefly, genomic DNA is extracted using a standard protocol, known to those in the art. PCR is run at 30 cycles: 93 °C x 30 seconds, 57 °C x 1.5 minutes, 72 °C x 5 minutes; using a sense primer ([Seq. Id. No.] SEQ ID NO: 7) and an antisense primer ([Seq. Id. No.] SEQ ID NO: 8).

Through the characterization of the amyloidogenic properties of β-synuclein and its mutants, and the elucidation of the association of β-synuclein with NACP/α-synuclein, the present invention provides a strategy for the testing and selection of therapeutic agents designed to negatively regulate the aggregation of NACP/α-synuclein *in vitro* through the manipulation of expression of non-amyloidogenic proteins.

Plasmid construction and preparation of recombinant proteins

Human NACP/α-synuclein, human β-synuclein, and its mutants, β-synuclein δ1 and β-synuclein δ2, are produced using the PROEX-1 6xHis expression system (GIBCO-BRL, Grand Island, NY) as previously described. The constructions of PROEX-1- NACP/α-synuclein and PROEX-1-β-synuclein are previously described (Hashimoto, *et al.*, *Brain Res.*, 799: 301-06, 1998). PROEX-1-β-synuclein δ1 is constructed by a two-step PCR protocol as follows:

Initially, primer pairs, either a sense primer BSYNNDE, encoding amino acids 1-7 of β-synuclein (SEQ ID NO: 1) and an NdeI site at its 5' end, and an antisense primer, corresponding to amino acids 73-83 of α-synuclein and amino acids 66-72 of β-synuclein (SEQ ID NO: 2), or a sense primer, corresponding to amino acids 73-83 of α-synuclein and amino acids 73-79 of β-synuclein (SEQ ID NO: 3) and an antisense primer BSYNNOT encoding amino acids 129-134 of β-synuclein and NotI site at its 5'end (SEQ ID NO: 4) are incubated in PCR with plasmid pCEP4-human β-synuclein as a template. Individual PCR products are gel-purified and an aliquot of each product is combed with primers BSYNNDE (SEQ ID NO: 1) and BSYNNOT (SEQ ID NO: 4) to synthesize a full length of β-synuclein δ1 cDNA. The resulting PCR product is digested with NdeI and NotI and ligated to PROEX-1 previously digested with NdeI and NotI, finally to produce PROEX-1-β-synuclein δ1.

PROEX-1-β-synuclein δ2 is similarly constructed by a two-step PCR protocol using PROEX-1-β-synuclein δ1 as a template. Initial primer pairs are

either BSYNNDE (SEQ ID NO 1) and an antisense primer corresponding to amino acids 55-62 of β-synuclein and amino acids 63-72 of α-synuclein (SEQ ID NO: 5) or a sense primer encoding amino acids 63-79 of α-synuclein (SEQ ID NO: 6), and BSYNNOT (SEQ ID NO: 4). For both of the newly-constructed plasmids, fidelity of the sequencing is confirmed using a commercially available delta taq cycle sequencing kit (Amersham).

In order to test the susceptibility of the newly constructed β-synuclein mutants to aggregation, an aggregation assay can be performed. To do this, 10-20 μM of each of the mutant proteins are incubated in a total volume of 20 μl with a final concentration of 100 mM sodium acetate, pH 6.9. Aggregation can be induced under a variety of conditions, for instance, low pH and high temperature. SDS-PAGE analysis/immunoblotting or Thioflavine-S staining provides means for evaluating the reacted samples, the methods of which are described above and well known to those in the art.

Histochemical analysis of β-synuclein mutants

In order to determine whether the aggregates resulting from aggregation reactions of PROEX-1-β-synuclein δ1 and PROEX-1-β-synuclein δ2 display amyloid-like characteristics, 10 μl aliquots of the protein preparations are obtained using the above-described procedures are pipetted onto glass slides and stained with Thioflavine-S. The results are visually analyzed using a confocal microscope, the method of which is well known to those in the art.

In vitro binding assay

Samples containing 0-20 μM of NACP/α-synuclein are incubated at 37 °C for 8 hours in combination with or without His-tagged β-synuclein (20 μM) in a total volume of 20 μl at pH 6.9. At the completion of the first incubation period, Ni^{2+} beads, equilibrated with buffer A (20 mM Tris-

HCl, pH 8.5, 100 mM KCl, 2 mM imidazole, 10 mM 2-mercaptoethanol and 10% glycerol), are added to the samples and left to sit for one hour at 4 °C. Following recovery of the beads by pulse centrifugation, the beads are washed three times with buffer A, once with buffer B (20 mM Tris-HCl, pH 8.5, 100 mM KCl, 20 mM imidazole, 10 mM 2-mercaptoethanol and 10% glycerol), and once again with buffer A. The beads are then boiled in SDS sample buffer, and subjected to 15% SDS-PAGE. NACP/α-synuclein is then visualized by the immunoblotting analysis described below.

Immunoblotting analysis using anti-NACP101-124 polyclonal antibody

NACP/α-synuclein-specific polyclonal antibody (anti-NACP101-124) is raised against the synthetic peptide 'GKGEEGYPDEGILEDMPVDPGSEA' (SEQ ID NO 9) which is derived from residues 101-124 of NACP/α-synuclein. Immunoblotting analysis is performed as described above, the method of which is well known to those in the art. Briefly, each sample is resolved by SDS-PAGE (15%) and blotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a standard transfer apparatus. The membrane is then blocked with TBS containing 3% BSA, followed by an incubation with anti-NACP101-124 (1:1000) in TBS containing 1% BSA. The treated membrane is then incubated with ^{125}I-labeled protein A (ICN, Costa Mesa, CA), followed by autoradiography.

The present invention also provides a method for examining the impact of abnormal accumulation of NACP/α-synuclein, resulting from wild type α-synuclein protein overexpression, on neuropathological alterations similar to those seen in Lewy body disease.

In order to better understand if the accumulation of α-synuclein results in neurodegenerative alterations similar to Lewy body disease, a line of transgenic mice were created in which wild type human α-synuclein is over-expressed under the regulatory control of the platelet-derived growth factor β (PDGFβ) promoter. The transgenic mice were

generated using the procedure described below. The PDGFβ promoter was chosen because it has been used successfully to target the expression of other human proteins to neurons in transgenic models of neurodegenerative disorders.

Human α-synuclein is a 140 amino-acid molecule (SEQ ID NO:10) that is encoded by a gene on chromosome 4 and was originally isolated from plaques of Alzheimer disease brains. Involved in synaptic function and neural plasticity, this 19 kD protein is a precursor of the highly hydrophobic 35 amino-acid metabolite, non-amyloid component (NAC). The NAC peptide can self-aggregate into fibrils and induces aggregation of the Aβ peptide.

Generation of transgenic mice

The transgene used for microinjection consists of a PDGFβ promoter, an SV40 intron, the human wild-type NACP/α-synuclein cDNA, and the SV40 poly A. Figure 7a illustrates the construct of the human PDGFβ promoter driven encoding human α-synuclein. This transgene is constructed by first subcloning the PDGFβ promoter into the EcoRI/XhoI site of pNASSβ using an EcoRI/XbaI linker. The portion of the β-gal cDNA and poly A signal in the resulting plasmid is substituted with the Not/SalI fragment of the pCEP4-NACP/α-synuclein which encodes human wild-type NACP/α-synuclein cDNA plus SV40 poly A cDNA. The purified construct is linearized by digestion with SalI/ScaI and injected into prenuclear mouse embryos from superovulated females and re-implanted in pseudo-pregnant female B6/DBA2 mice. The offspring genotype is confirmed by slot blot, the procedure of which is well known to those in the art.

Thirteen founder mouse lines are generated and crossed with wild-type, B6/DBA2 mice. The resulting mice from each line are screened by PCR using tail DNA. Briefly, genomic DNA is extracted using a standard protocol, known to those in the art. PCR is run at 30 cycles: 93 °C x 30

seconds, 57 °C x 1.5 minutes, 72 °C x 5 minutes; using a sense primer (SEQ ID NO: 7) and an antisense primer (SEQ ID NO: 8).

Offspring from each line are sacrificed in order to determine the levels of mRNA expression, using ribonuclease protection assay (RPA) analysis, and protein expression using Western blot and immunochemical analysis. After the initial screening and characterization, the mouse lines that show high, intermediate, and low levels of transgene expression are selected for subsequent breeding and neuropathological analysis.

Tissue processing

Heterozygous mice from transgenic lines expressing high, intermediate and low level of NACP/α-synuclein, as well as non-transgenic littermates are perfused with cold saline and the brains removed, intact. The left hemibrain is frozen with isopentane and cooled in a Histobath (Shandon Lipshaw, Pittsburgh, PA) and the right hemibrain is immersion-fixed in 4% paraformaldehyde in pH 7.4 phosphate buffered saline (PBS). The frozen samples of the left hemibrain are bisected and processed for RPA, Western blot and dot blot analysis. The immersion-fixed right hemibrains are serially sectioned into 40 μm slices with the Vibratome 2000 (Leica, Deerfield, IL) for subsequent neuropathological, immunocytochemical/computer aided imaging and ultrastructural analysis. An additional subset of paraformaldehyde-fixed brains is paraffin-embedded and serially sectioned at 7μm for H & E and Cresyl violet staining and *in situ* hybridization.

Analysis of NACP/α-synuclein transgene mRNA expression

For the RPA analysis, the human NACP/α-synuclein/SV40 riboprobe is generated by PCR using primers located in the PDGF-h NACP/α-synuclein transgene. The sense primer is a 20- mer starting at nucleotide no. 194 of h NACP/α-synuclein (GenBank Accession #L08850). The anti-sense primer is a 21- mer starting at nucleotide no. 270 of the SV40 poly